THE GROUPING OF A SERIES OF CHLOROACETANILIDE PESTICIDES BASED ON A COMMON MECHANISM OF TOXICITY

[This case study does not represent a regulatory decision by the EPA. Rather, it attempts to demonstrate a proposed process by which pesticide chemicals can be evaluated.]

I. INTRODUCTION

The Food Quality Protection Act of 1996 (FQPA) requires EPA to perform a combined risk assessment for chemicals that produce adverse effects by a common mechanism of toxicity. Central to performing this task is the process of identification of those pesticide chemicals that can be grouped based on a common mechanism of action.

This document attempts to apply the principles outlined in the accompanying guidance document on common mechanisms of toxicity to determine if a group of pesticide chemicals (chloroacetanilide pesticides) share a common mechanism of toxicity.

Although data submitted under FIFRA testing guidelines include extensive toxicological information, only in a few cases is mechanistic information available. Thus, selection of the group of chloroacetanilide pesticides with a possible common mechanism of action cannot be done at this time by simple side-by-side comparison of already elucidated mechanisms of action. Instead, commonality of mechanism of action will be inferred based on considerations from:

- o Structure-Activity relationships: Selection of chemicals based on their likelihood to generate a common type of reactive intermediate
- o Toxicology: Selection of chemicals with common toxic effects
- o Metabolism and Pharmacokinetics: Selection of chemicals based on similarities of disposition and on their generation of common types of reactive metabolites.

II. THE CANDIDATE SERIES OF PESTICIDES

For the purposes of this case study, the group of compounds shown in Figure 1 was selected as the candidate series of chloroacetanilide pesticides, that is, they were selected based upon structural similarity. This group initially was selected based upon them all possessing the chloroacetanilide moiety. It is noted that Chlor-7, with the amino group attached to a thiophene ring rather than to a benzene ring, is not a chloroacetanilide. However, the compound was included because it still contained the chloroacetyl amino moiety attached to an aromatic ring.

III. ELEMENTS OF EVIDENCE

In this section, the various elements of available evidence for the compounds under evaluation will be presented. These elements will be used in the weight-of-evidence evaluation.

A. Structure Activity Considerations

In general, based on the use of structure-activity relationships (SAR), the pesticides in a given mixture may be grouped according to their likelihood to generate a common type of reactive intermediate or their ability to mimic a common biologically active molecule to interfere with the normal homeostasis of the cell (e.g., via receptor binding, enzyme induction, etc.) to exert toxic action.

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For the pesticides in this Case Study, at least four reactive intermediates capable of eliciting toxic action may be identified. These include: (a) the active chlorine þ to the carbonyl group, (b) reactive quinone imine intermediate, (c) formaldehyde, (d) α,β–unsaturated aldehyde.

All seven pesticides in this Case Study contain the direct-acting active chlorine α to the carbonyl group. This type of electrophilic reactive compounds are expected to preferentially react with "soft" nucleophiles such as glutathione (GSH) and SH-containing proteins. All seven pesticides are therefore expected to react with GSH to cause depletion of the protective nucleophile. Such depletion is expected to be particularly sensitive for tissues with relatively low level of endogenous GSH (e.g., blood, nasal tissue, stomach) rendering them more susceptible to the toxic action of this or other types of reactive intermediates. Alternatively, these pesticides may directly react with SH-containing proteins at or near the port of entry, or initial site of absorption (e.g., blood) to exert toxic action.

With the exception of Chlor-7, all the pesticides in the Case Study are *potential* substrates for generating the reactive quinone imine intermediate after N-dealkylation (by mixed function oxidase) and N-deacylation (by aryl amidase) of the pesticides with subsequent ring hydroxylation and oxidation. However, since N-dealkylation requires α–hydroxylation, Chlor-5 and Chlor-6 are expected to be substantially poorer substrates because of steric hindrance at the α–carbon. Likewise, the presence of a hydrophilic carboxylic acid group adjacent to the α–carbon is also expected to render Chlor-4 a poor substrate. Thus, only Chlor-1, Chlor-2 and Chlor-3 are expected to be relatively good substrates for generating the quinone imine intermediate. The quinone imine intermediate, once formed, is capable of reacting with macromolecules, particularly in tissues in which endogenous GSH is depleted.

Three pesticides in the Case Study -- Chlor-1, Chlor-2 and Chlor-3 -- are potential generators of formaldehyde. Metabolic O-dealkylation of the terminal alkyl group is expected to yield the unstable N-methylol moiety which can spontaneously decompose to yield formaldehyde. Alternatively, there is also some possibility that the methylene (-CH_2-) group between the two heteroatoms (N,O) may be susceptible to acid hydrolysis to yield formaldehyde. Formaldehyde, once formed, may be rapidly detoxified or serve as crosslinking agent to initiate toxic action at or near the site of generation. Exposure to high doses of formaldehyde has been associated with the induction of nasal tumors. *In situ* metabolic production of formaldehyde has been postulated to be the most likely reactive intermediate in the nasal carcinogenic action of hexamethylphosphoramide (HMPA).

Chlor-7 is unique among the pesticides in the Case Study in its ability to undergo side chain oxidation to yield an α,β–unsaturated aldehyde as a reactive intermediate. A number of α,β–unsaturated aldehydes, such as furfural or acrolein, have been shown to be carcinogenic or genotoxic.

Thus, based on SAR considerations of common reactive intermediates, only Chlor-1, Chlor-2 and Chlor-3 seem to share a common mechanism of action in several aspects. In addition, depending on the type of tissue involved, all seven pesticides may have a common mechanism of action IF tissue depletion of GSH is a critical factor.

Much less is known about the ability of the pesticides in this Case Study to mimic a common biologically active molecule by interfering with the normal homeostasis of the cell to exert a toxic action. There is some evidence that several pesticides in this group are capable of inducing mixed-function oxidases; however, there is no evidence to relate any structural moieties or features to their ability to bring about enzyme induction.

B. Toxicological Considerations

Table 1 summarizes toxic effects observed in chronic studies with the candidate chloroacetanilides. Effects common to 3 or more chemicals were seen in nasal tissue, thyroid, stomach, kidneys, and liver.

o **Nasal Tissue** - Statistically significant increases in nasal tumors have been reported for Chlor-1, Chlor-2 and Chlor-3 in rats. Additionally, nasal tumors (1 adenocarcinoma and 1 fibrosarcoma) have been reported in rats fed Chlor-5 at 3000 ppm in the diet. Although the results did not reach statistical significance for Chlor-1, nasal turbinate tumors are considered to be rare and these results are suggestive of a neoplastic response at that site. Nasal tumors have not been reported for other compounds in Table 1. No nasal tumors have been reported for mice.

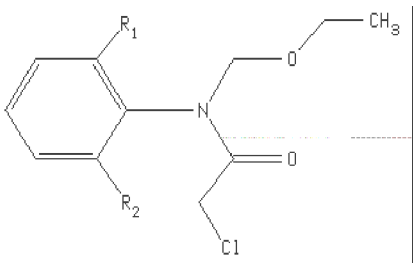
o **Thyroid Gland -** Thyroid follicular cell tumors have been reported for Chlor-1, Chlor-2, and Chlor-3 in rats. Additionally, in Sprague-Dawley (SD) rats tested with Chlor-6 at doses of up to 500 ppm in the diet for 104 weeks, the incidence of thyroid neoplasia appeared to be increased, but remain within historical control levels. Thyroid tumors have not been reported for other compounds in Table 1.

o **Stomach -** Stomach tumors have been reported for Chlor-1, Chlor-2 and Chlor-3 in rats. Male S-D rats administered Chlor-7 in the diet for 104 weeks showed trend and pairwise statistically significant epithelial hyperplasia of the stomach. Stomach lesions have been reported in CD-1 mice of both sexes administered Chlor-6 in the diet for 18 months at levels of 0, 100, 500, 1500 or 6000 ppm. Herniated mucosal glands into the submucosa/tunica muscularis were observed in both sexes at the highest dose and in some males at the next highest dose level. Males at the highest dose level also showed erosion/ulceration of the glandular mucosa of the stomach.

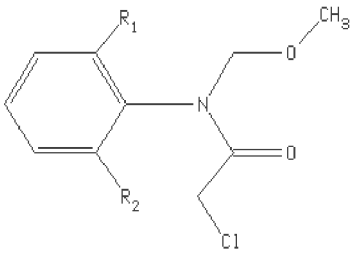
o **Kidney** - Kidney effects have been reported for Chlor-1, Chlor-2 and Chlor-3. Chlor-1 showed histopathology in a 1-year dog study, increased relative kidney weights in a 2-year rat study and in a 78-week mouse study. Chlor-2 has shown elevated BSP values in a 1-year dog study and increased kidney sclerosis in a 2-year rat study. Chlor-3 has shown chronic nephropathy in the rat in a 2-year study, in addition to kidney tumors.

o **Liver** - Liver effects have been reported for most chemicals of the candidate series.

Chlor-1, Chlor-2, Chlor-3, Chlor-6, and Chlor-7 have produced increases in relative liver weights, coupled in some cases with hepatocellular hypertrophy. These changes are consistent with an induction of microsomal enzymes. In fact, experimental data exist to indicate that Chlor-2 induces microsomal enzymes (hepatic T4-UDPGT activity in rats is induced by Chlor-2 to 168-194% of control levels after repeated administration).



Chlor-1



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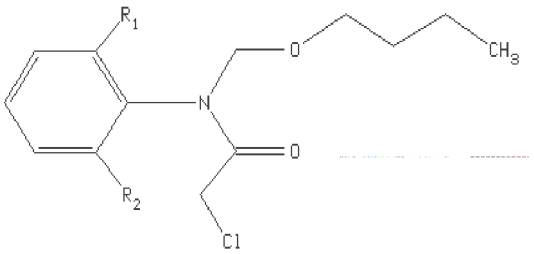
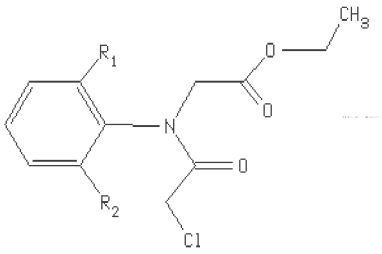
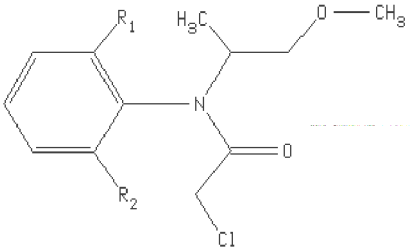


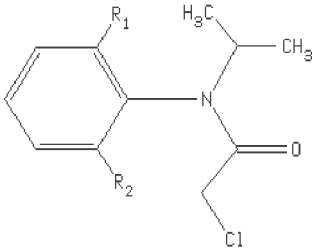
Chlor-3



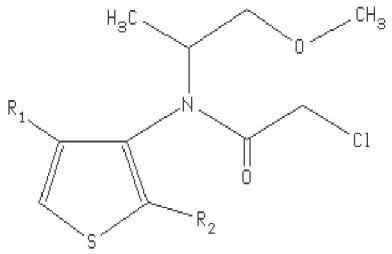
Chlor-4



Chlor-5



Chlor-6



Chlor-7

Figure 1. Structures of the Candidate Set of Chloroacetanilides, R_1 and R_2 are H or alkyl.

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Table 1. Toxicology Data for the Candidate Set of Chloroacetanilide Pesticides

Effects	Chlor-1	Chlor-2	Chlor-3
Nervous System	1-year dog: brain histopathology (50 mg/kg/d), salivation and neurotoxic signs (10 mg/kg/d).	2-Year rat: Compression atrophy of the brain 14 mg/kg/day	-
Renal System	1-Year dog: kidney histopathology (40 mg/kg/day). 2-Year rat: kidney rel. wts. þ (79.6 mg/kg/d) - 78 Week mouse: absolute and relative kidney (þ, dose-related) tubular basophilia (þ).	1-Year dog: BSP þ (10 mg/kg/d) 2-Year rat: tubular sclerosis kidney þ (15 mg/kg/d) 18-Month Mouse: slight increases in tubular epithelium hyperplasia/regeneration in males.	2-Year rat : Chronic nephropathy increased (5 mg/kg/d). Kidney cortical tumors at 150 mg/kg/d). 2-Year rat: Tubular cell hyperplasia & pelvic epithelial hyperplasia (40 mg/kg/d). No tumors.
Hematology/Clinical Chemistry	1-Year dog: Cholesterol (þ), 50 mg/kg/d) 2-Year mouse: RBC, Hct and Hb (þ) at greater than 500 ppm	1-Year dog: Hemolytic anemia (3 mg/kg/d) in males; Hemosiderosis in kidney, liver, and spleen of males only.	1-Year dog: Cholesterol (þ), 25 mg/kg/d. 90-Day rat: Mild anemia plus spleen hemosiderosis at 3000-5000 ppm
Ovary/Testes	1-Year dog: testes weights þ, tubular degeneration, hypospermia (40 & 50 mg/kg/d)	2-Year rat: ovarian wt þ (15 mg/kg/d)	-
Eye	2-Year rat: ocular lesions (79 mg/kg/d) 2-Year mouse: positive trend for retinal degeneration (1500 & 5000 ppm) 78-Week mouse: increased (not significantly) lens vacuolation at $\geq$100 ppm	2-Year rat: ocular lesions (uveal degeneration, 14 mg/kg/d plus corneal opacity at 42 mg/kg/d). 2-Year rat: ocular lesions (uveal degeneration, 15 mg/kg/d)	2-Year rat: cataract & retinal atrophy significantly increased in females vs controls at 40 mg/kg/d
Liver	Increased relative liver weights in dog, rat and mouse chronic studies. 78-Week mouse: significant increase in combined hepatocytic adenomas plus carcinomas observed in males only at the high dose.	6-Month dog: increased relative weight, 15 mg/kg/d, fatty degeneration/biliary hyperplasia, 25 mg/kg. 1-Year dog: Significantly increased absolute and relative weights in males only; values in females were increased but not significantly. 18-Month mouse: Hepatocellular hypertrophy in males only.	1-Year dog: hepatocellular swelling and increased liver weight (25 mg/kg/d). 2-Year rat: significantly increased incidence of hepatocellular swelling, acidophilic foci and mixed cell foci of alteration in males.
Stomach	2-Year rat: basal cell tumors (limited to 1þ and 1þ at the high dose).	2-Year rat: significant increasing trends and significant pair-wise increases in malignant mixed gastric tumors and gastric adenocarcinoma and/or mixed gastric tumors combined in both sexes	2-Year rat: Tumors limited to females. Significant increasing trends and pairwise comparison for carcinomas, carcinosarcomas, and combined carcinosarc./leiomyosarcomas. Significant increasing trend for leiomyosarcomas.
Thyroid	2-Year rat: follicular cell tumors, significant positive trend in both sexes and pairwise significant increases in adenomas and combined adenomas/carcinomas at the high dose in females only.	2-Year rat: significant increasing trends and significant pair-wise increases in thyroid follicular cell adenomas, carcinomas and combined adenomas/carcinomas in males and significant increasing trends in thyroid follicular cell adenomas and combined adenomas/adenocarcinomas in females.	2-Year rat: Significant increasing trend in follicular cell adenomas and adenomas and/or carcinomas combined in males and significant increasing trend in follicular cell adenomas, carcinomas and adenomas and/or carcinomas combined in females; plus pairwise increases in follicular cell adenomas and adenomas and/or carcinomas combined in both sexes.
Nasal Tissues	2-Year rat: significant pairwise increases in nasal epithelium adenomas were seen for both sexes at the high-dose vs. controls ($p \leq 0.01$ for trend and pairwise comparisons). Carcinomas of the nasal epithelium, although not statistically significant, had an incidence of 3% (þ) and 2% (þ) at the high dose only vs 0% in controls. There were statistically significant trends in carcinomas alone and combined carcinoma adenoma for both sexes.	2-Year rat: Significant increasing trends and pair-wise increases in nasal respiratory epithelium adenomas and combined adenomas/carcinomas in both sexes were observed in two studies.	2-Year rat: In males: Significant increasing trends and pair-wise increases in nasal respiratory/olfactory epithelium adenomas and combined adenomas/carcinomas; in females: significant increasing trends in nasal respiratory/olfactory epithelium adenomas, carcinomas and combined adenomas/carcinomas plus pair-wise increases in nasal respiratory/olfactory epithelium adenomas, and combined adenomas/carcinomas.

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Table 1. Toxicology Data for Chloroacetanilide Pesticides (Continued)

Effects	Chlor-4	Chlor-7	Chlor-5	Chlor-6
Nervous System	No Data	No	No	No
Renal System	No Data	No	No	No
Hematology/Clinical Chemistry	1-Year Dog: Pos. Coombs (0.25 mg/kg/d); Cholesterol þ (31 mg/kg/d)	1-Year dog: Cholesterol (þ transient) 13-Week dog: Cholesterol (þ dose-related). 2-year rat: Cholesterol (þ) at high dose. 90-day rat: Cholesterol (þ dose-related)	No	No
Ovary/Testes	No Data	No	No	* No
Eye	No Data	2-Year rat: Treatment-related exacerbation of posterior lenticular opacity (age-related effect)	No	No
Liver	Liver vacuolation	1-Year dog: hepatocellular vacuolation, mid-zonal hepatocytic enlargement. 2-year rat: Increased relative liver weights statistically significant trend for hepatocellular adenomas/carcinomas in males; trend and pairwise increase in altered eosinophilic hepatocytes. 94-Week Mouse: Statistically significant increases in relative liver weights and dose related hepatocyte enlargement.	2-year rat: significant increasing trend in liver neoplastic nodules (adenomas) and combined adenomas and carcinomas in both sexes. Significant pairwise increases in liver adenomas and combined adenomas/carcinomas in females.	18-Month mouse: Dose-related increases in relative liver weights and in hepatocellular hypertrophy in both sexes. Additionally there was necrosis of individual hepatocytes, eosinophilic foci, teleangiectasis. There was a statistically significant increase in hepatocellular tumors [adenomas, carcinomas, hepatoblastomas] in high dose males.
Stomach	No Tumors	2-Year rat: trend and pairwise increase in and epithelial hyperplasia of the stomach in males. 94-Week mouse: Stomach hyperkeratosis at the limiting ridge in both sexes.	No Tumors	18-Months mouse: herniated mucosal glands, erosion ulceration of the glandular mucosa.
Thyroid	No Tumors	No Tumors	No	104-Week rat at doses up to 500 ppm: the incidence of thyroid neoplasia appeared to be increased, but remained within historical control levels.
Nasal Tissues	No Tumors	No Tumors	2-Year rat : Nasal tumors were not statistically significantly elevated but 1 adenocarcinoma (nasal gland) and 1 neurofibrosarcoma (peripheral nerve) were seen in high-dose males vs 0 in controls. Polipoid adenomas of the respiratory epithelium were seen in controls (1) and high dose males (1) and in mid-dose females (1). A squamous papilloma was seen in high-dose females and none in controls. Dosing is considered to marginally adequate for carcinogenicity assessment.	No

Chlor-6 has also been found to decrease the barbiturate-induced sleeping time in rats.

Chlor-5, Chlor-6 and Chlor-7 have produced liver tumors in rodents in chronic studies. Chlor-7 has produced a statistically significant trend for hepatocellular adenomas/carcinomas in rats. Chlor-5 produced statistically significant increases in liver adenomas and combined adenomas/carcinomas in female rats and a statistically significant trend for liver tumors in male rats. Chlor-6 produced a statistically significant increase in hepatocellular tumors [adenomas, carcinomas, hepatoblastomas] in high dose male CD-1 mice.

C. Metabolism and Pharmacokinetics Considerations

In general, metabolism and pharmacokinetics considerations are important in the inference of common mechanisms of action in a candidate set of toxic chemicals. The study of the disposition of a chemical helps to elucidate issues of dose delivery to the target sites: delivery to the target site is a prerequisite for toxicological activity at the site. The study of the biotransformation of the chemicals will determine if a putative common toxic species or its precursor are produced.

Chemicals in the candidate set have similarities in disposition. There are no metabolism data for Chlor-4, thus the compound is not discussed in this section.

a. Absorption - Absorption of these chemicals after oral dosing is almost complete or at least very extensive. Data for Chlor-6 indicate that at least 68% of the dose is absorbed after oral dosing; absorption of the other compounds reaches 90% of the dose or more.

b. Tissue Distribution - Following oral dosing, radioactivity from the [C^{14}]-labeled parent and/or its metabolites is distributed extensively through all major organs in rats.

The radioactivity is seen to bind extensively (up to 3% of the dose) to **red blood cells (RBC)** producing blood/plasma ratios of 18 to 315 for Chlor-1, Chlor-2, and Chlor-3. Likewise, Chlor-5 produced RBC/liver protein ratios greater than 11. The nature of the bound material is not known.

In the case of Chlor-2, levels of radioactivity in the non-glandular **stomach** exceeded those in the glandular stomach. As the dose decreased, the non-glandular stomach showed a decrease in percent of dose present, while the glandular stomach showed minor decreases in percent of dose of Chlor-2 derived radioactivity.

Studies using WBA indicate that radioactivity from radiolabeled Chlor-2 and Chlor-5 is distributed to the **nasal turbinates** in the Long-Evans rat. Additional experiments with radiolabeled Chlor-2 indicate that distribution to the nasal turbinates is strain and species specific to the rat and is not observed in mice, hamsters or squirrel-monkeys. In fact,

- Female Long-Evans rats, female CD-1 mice, and male squirrel monkeys were dosed with single oral doses of [^{14}C]Chlor-2 at levels of 7, 70 or 700 mg/kg. Using whole body autoradiography (WBA), radioactivity was found in liver, kidney, nasal vibrissae, body hair, oral structures, and periorbital fat of all species. At 5 days, accumulation of labeled material was significant in the nasal turbinates of the rat, less in the mouse, and absent in the squirrel monkey.

- Similarly, female Sprague-Dawley, Long-Evans, and Fisher 344 rats and female Syrian hamsters were given single oral doses of [^{14}C]Chlor-2 at 7 or 70 mg/kg and tissue distribution was studied by WBA. At 24 hours post dosing all three strains of rat showed radiolabel in the highly perfused tissues. Nasal localization was observed in all three strains, but was most apparent in the Long-Evans strain. At no time was there any label in the nasal tissues of the Syrian Hamster.

- Single oral doses of 0.7 or 7.0 mg/kg [^{14}C]Chlor-2 methylsulfide (a metabolite of Chlor-2) given to female Long-Evans rats and the tissue distribution of radioactivity was studied by WBA. At 1-day post dose, radioactivity was observed in the intestines, stomach, and nasal turbinates. At 5 days localization of [^{14}C] was still evident in the nasal turbinates.

- Single oral doses of 7 or 70 mg/kg [^{14}C] dialkylaniline (metabolite of Chlor-2) were given to female Sprague-Dawley and CD-1 mice. At one day after dosing radioactivity was present in the major tissues of both rats and mice. Nasal localization was evident in the rat but not the mouse.

c. Excretion - Following oral dosing of rats with [C ^{14}]-labeled chemical the ratio of urine/feces excretion varies among the compounds, reflecting the differences in tissue distribution and metabolite disposition among the compounds. Thus, the ratio of urinary excretion to feces excretion is close to 1 for Chlor-2, Chlor-5 and for low doses of Chlor-7, greater than 1 for Chlor-1, Chlor-6 and high doses of Chlor-7, and less than 1 for Chlor-3.

A large fraction of an oral dose of these compounds is conjugated and excreted to the intestine via the bile. While in the intestine, these conjugates excreted in the bile undergo further biotransformation followed by partial reabsorption through the intestinal wall, constituting a cyclic process of enterohepatic circulation. This process of enterohepatic circulation is a significant mechanism in the generation and the distribution of toxic metabolites.

d. Biotransformation - All members of the candidate series undergo extensive biotransformation in rats. Amounts of untransformed parent compound range from undetectable to 8% or less in feces. Numerous metabolites have been detected in numbers ranging from at least 11 in urine of Chlor-6-treated rats up to 40 in urine of Chlor-3-treated rats.

As expected from SAR considerations, all members of the candidate series undergo glutathione conjugation at the chloroacetyl group, N-dealkylation, oxidative metabolism of the N-alkyl group, and in some cases oxidative metabolism or the ring alkyl groups.

i. **Glutathione Conjugation and Quinone Imine Formation -** Glutathione conjugation at the chloroacetyl group is the major pathway of biotransformation for the compounds of the candidate series. Glutathione conjugation is of importance in interpreting the toxicity of these compounds because one of the products of further biotransformation of the glutathione conjugate of Chlor-2, **the electrophile 3,5-dialkylbenzoquinone-4-imine (DABQI)**, has been associated with the production of nasal tumors in the rat.

The current working hypothesis for the induction of nasal tumors in rats exposed to Chlor-2 by the oral route proposes that Chlor-2 conjugates with glutathione and is excreted in the bile. Subsequent biotransformation of the conjugate to a series of sulfur-containing products followed by enterohepatic circulation of these products creates a pool of metabolites that are delivered

to the nose where they undergo further metabolism to toxic materials. Metabolism by nasal enzymes results in formation of **DABQI** (Figures 2 and 3) which binds to cellular proteins, producing cytotoxicity and eventual neoplasia.

As shown in Figure 2, a methyl sulfide metabolite of Chlor-2 is converted to 2,6-dialkylaniline (2,6-DAA) following arylamidase action. 2,6-DAA is activated via a **phenol metabolite precursor (4-amino-3,5-dialkylphenol)** to DABQI. Although the 4-amino-3,5-dialkylphenol has not been identified in excreta of rats dosed with Chlor-2, this phenol has been formed <u>in vivo</u> in Long-Evans rats dosed with the methyl sulfide metabolite of Chlor-2 (Figure 2), appearing in urine as the sulfate (Figure 4) in quantities of 0.9-1.7% of the dose.

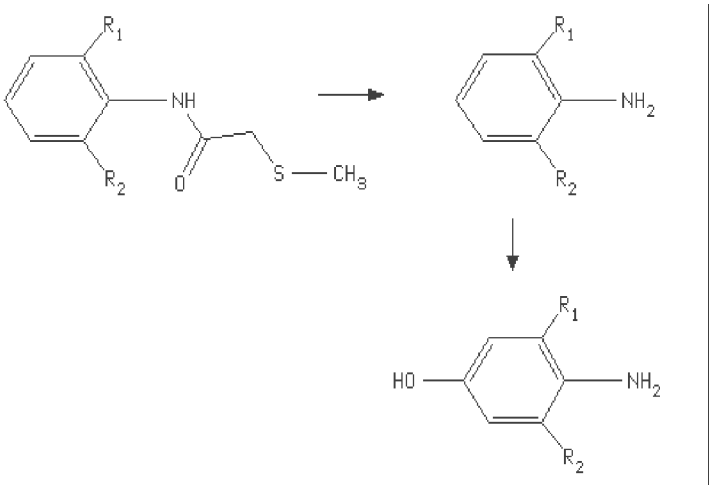

Figure 2. Formation of a 4-aminophenol derivative from a methylsulfide metabolite of Chlor-1. R_1 and R_2 are H or alkyl groups.

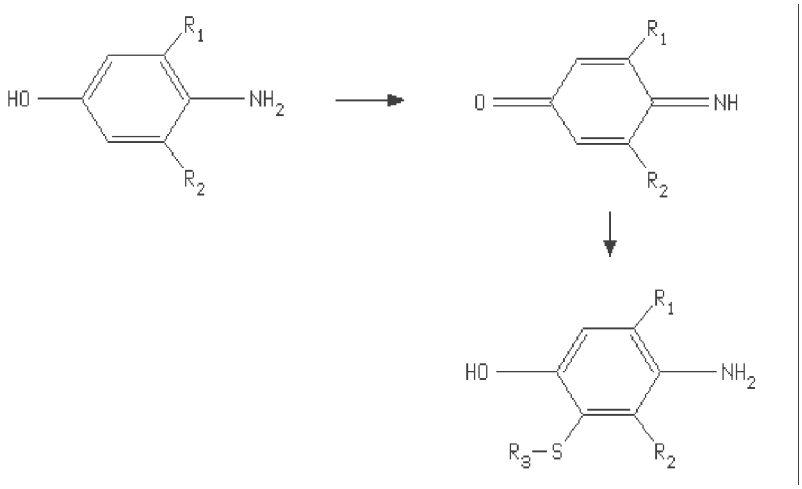

Figure 3. Quinone imine formation from a 4-aminophenol derivative of Chlor-1. R_1 and R_2 are H or alkyl groups. R_3-S: nucleophile attached to the quinoneimine

Data for the other members of the candidate series indicate that some of them, Chlor-3 and Chlor-6, are metabolized to the corresponding 4-aminophenol derivative, precursor of a quinone imine.

In the case of **Chlor-3**, although no 4-amino-3,5-dialkylphenol or its sulfate were identified after oral dosing of rats, **4-amino-3,5-dialkylphenol sulfate** (Figure 4) has been identified in urine and feces of SD rats dosed iv with a single dose of [^{14}C]Chlor-3 at 1, 10, or 100 mg/kg. Levels in urine were 1.9-2.5% of the dose in males and 1.1-2.0% of the dose in females. 4-Amino-3,5-dialkylphenol can be activated to the quinone imine shown in Figure 3. It is noted that its sulfate conjugation product, 4-amino-3,5-dialkylphenol sulfate, was sought but not found in urine or feces of rhesus monkeys.

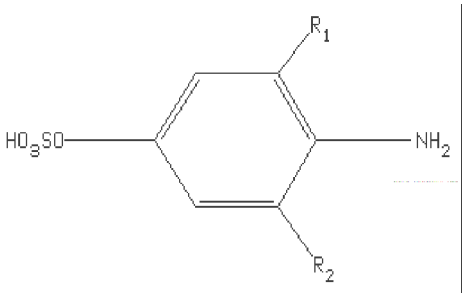

Figure 4. 3,5-Dialky-4-aminophenol sulfate, R_1 and R_2 are H or alkyl.

In the case of **Chlor-6**, the N-acetyl derivative of the 4-aminophenol derivative and its glucuronide were found in urine of rats dosed orally with Chlor-6. Data in the literature indicate that N-acetyl-4-aminophenol can be activated to a quinone imine and has been found to cause necrosis in rat and human liver.

In the case of **Chlor-1**, the mercapturic acid conjugate of N-de-alkylated Chlor-1 (Figure 5), comprising 22-32% of the dose, is metabolized to a methyl sulfoxide which presumably has originated from a putative Chlor-1 methyl sulfide intermediate (not in the figure). **By analogy with Chlor-2, this putative S-methyl compound could also be convertible to dialkylaniline, hydroxylated and then activated to a quinone imine. However, no actual data on this conversion is available at this time.**

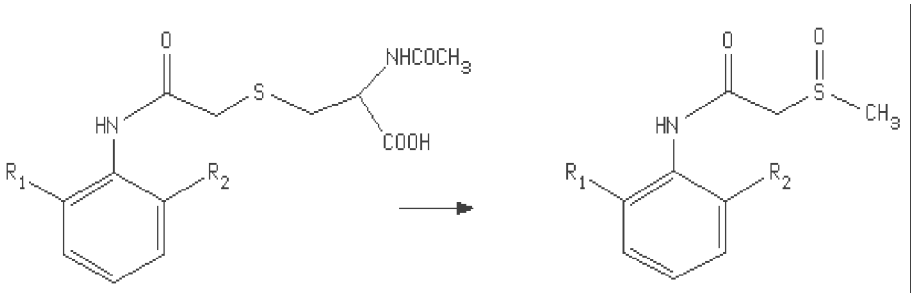

Figure 5. Sulfoxide formation from a mercapturic acid derivative of Chlor-1, R_1 and R_2 are H or alkyl.

In the case of **Chlor-5**, 2,6-dialkylaniline (a possible quinone imine precursor) has been detected, however it has been detected at less than 0.00055% of the dose.

In the case of Chlor-7, the formation of the p-quinone imine is not structurally possible. There is no data for Chlor-4.

ii. Other Reactions - As discussed above, metabolic products include dealkylated materials resulting in secondary amines. The toxicological significance of these materials is not clear.

IV. MECHANISMS OF THE TOXIC EFFECTS

This section depicts some of the postulated mechanisms for some of toxic endpoints by which the chemicals of this case study might be grouped. In particular, these sections review tumorigenesis in nasal turbinates and the thyroid, as studied with Chlor-2, and mechanisms of stomach tumorigenesis from Chlor-3. No mechanism is suggested as causing liver toxicity, however, the combination of SAR and the profile of adverse effects in the liver are presented as another possible mode of grouping.

A. Mechanistic Aspects of Nasal Turbinate Tumorigenesis in Rats

This Section summarizes the postulated mechanism for the formation of nasal tumors as investigated for **Chlor-2.**

The mechanism whereby chloroacetanilide herbicides produce nasal tumors in rats has been extensively investigated for Chlor-2.

In the rat, Chlor-2 is metabolized to the glutathione conjugate which is excreted into the gut through the bile. In the gut, enteric bacteria metabolize the conjugate to the thiol conjugate, with subsequent S-methylation of the thiol. The product of this reaction, the methyl sulfide, is re-absorbed into the systemic circulation where conversion to the secondary sulfide occurs. Hydrolysis of the secondary sulfide by arylamidase produces the dialkylaniline metabolite of Chlor-2. Oxidation of the dialkylaniline metabolite produces the putative toxic metabolite 3,5-dialkylbenzoquinone-4-imine (DABQI). DABQI binds to cellular protein resulting in eventual cell death. Ensuing regenerative cell proliferation can then lead to neoplasia through fixation of spontaneous neoplasms. Production of a quinone imine metabolite has been demonstrated directly in the case of Chlor-2. While not verified directly, data for Chlor-2 show binding of the DABQI metabolite to nasal protein at doses which deplete hepatic glutathione and which cause nasal tumors, supporting a non-genotoxic mode of action for nasal tumorigenicity.

Evidence for the metabolic formation of a quinone imine metabolite for other members of this Case Study group was discussed above.

B. Mechanistic Aspects of Stomach Tumors in Rats

This Section summarizes the postulated mechanism for the formation of stomach tumors as investigated for **Chlor-3.**

Mechanistic aspects of stomach tumor formation as a result of chloroacetanilide administration to rats have been derived largely from studies conducted on Chlor-3.

Data reviewed for Chlor-3 show that at a high dose of 213 mg/kg/day given to rats for 22 months, cell proliferation in the neck and base regions of the fundus glands of the stomach was significantly increased, while mucosal thickness was decreased in relation to untreated rats. Gastric pH and serum gastrin levels were also increased at the high dose. Stomach tumors were observed, with small (early) tumors composed of enterochromaffin like

endocrine cells of glandular tissue. Larger tumors (late neoplasms) were of mixed cell types, with some predominantly endocrine-like and others containing endocrine like cells which were poorly differentiated. Advanced tumors may have progressed from well differentiated neuroendocrine lesions to more undifferentiated neoplasms, or there may have been more than one cell type of origin for a tumor.

Based on the above data, the mechanism proposed for stomach tumor formation involves atrophy of the fundic mucosa following high dose exposure and consequent loss of the deeper elements of the mucosal epithelium. Mucosal atrophy leads to compensatory cell proliferation in the fundic mucosa, while loss of parietal cells results in extensive gastric hypochlorhydria and a subsequent increase in gastric pH. The increase in gastric pH induces excessive production of gastrin, resulting in elevated serum gastrin. The trophic effect of gastrin on the enterochromaffin-like and fundic stem cells further drives a sustained cell proliferation which ultimately results in induction of gastric neoplasms.

C. Mechanistic Aspects of Thyroid Follicular Cell Tumors

This Section summarizes the postulated mechanism for the formation of thyroid follicular tumors as investigated for **Chlor-2.**

The mechanistic information in support of thyroid tumor induction is derived from data for Chlor-2. The mechanism whereby thyroid tumors arise in rats from the chronic administration of Chlor-2 appears to be based on the induction of hepatic uridine 5'-diphospho glucuronyltransferase (UDPGT) with a subsequent decrease in circulating T3 and T4 levels, a subsequent increase in circulating TSH, and eventual hyperplasia and neoplasia of the thyroid based on exposure of the rat thyroid to elevated and sustained levels of TSH. A mechanistic study conducted by the registrant for Chlor-2 included 5 groups of rats which were administered Chlor-2 in the diet at 126 mg/kg/day for up to 120 days. An additional group of 20 rats was exposed to Chlor-2 for 60 days and then untreated diet for another 60 days to determine reversibility. The results of this study showed consistent increases in liver weight, thyroid weight, and activity of UDPGT as well as elevations in serum TSH. Changes in T3 and T4 were inconsistent. Elevations in TSH, liver weight, and thyroid weight were reversible upon cessation of exposure. The data from this study show that Chlor-2 appears to act in a manner similar to that observed with a wide range of chemicals which are inducers of hepatic microsomal enzymes, but which are not mutagenic and produce neoplasia only at high doses.

VII. Weight of the Evidence

Table 2 lists the various parameters that are considered to be relevant in defining those chemicals that can be considered to have a common mechanism of action.

[This case study does not represent a regulatory decision by the EPA. Rather, it attempts to demonstrate a proposed process by which pesticide chemicals can be evaluated.]

Table 2. Evidence used in grouping/excluding chloroacetanilide pesticides by common mechanism of action. [a]

Parameter	Chlor-1	Chlor-2	Chlor-3	Chlor-5	Chlor-4	Chlor-7	Chlor-6
Nasal tumors in rats	Yes	Yes	Yes	Yes, but p>0.05. Chemical distributes to nasal turbinates in rats	No	No	No
Forms quinone imine (QI) metabolite	Yes (inferred from identification of precursor)	Yes	Yes (inferred from identification of precursor)	Possible (levels of 2,6 -dialkylaniline are very low)	No data	Unable to form	Probably. Forms the p-aminophenol derivative, which can be activated to a QI
Nasal tumors based on QI	Likely, based on similar nasal profile as Chlor-2	Yes	Possible	Nasal tumors, but not statistically significant	No	No	No
Thyroid tumors in rats	Yes	Yes	Yes	No	No	No	Increased (not stat. significantly), but within historical controls
Thyroid tumors based on induction of hepatic microsomal enzymes	Possible; no direct evidence	Yes	Possible, no direct evidence	No	No	No	Not known. But compound decreases barbiturate-induced sleeping time in rats
Liver Tumors	Statistically significant increase in the incidence of combined hepatocytic adenomas/carcinomas was observed in male mice only at the high dose	No	No	Liver adenoma/carcino. in rats: trend and pairwise in females, trend in males	No	Trend ($p \leq 0.05$) for liver tumors in rats,	Significant increase in hepatocellular tumors at the high dose in male mice

[This case study does not represent a regulatory decision by the EPA. Rather, it attempts to demonstrate a proposed process by which pesticide chemicals can be evaluated.]

A. TOXICOLOGICAL EVIDENCE

1. Nasal tumors

Three members of the candidate series (**Chlor-1, Chlor-2, and Chlor-3**) show tumors of the nasal turbinates, additionally **Chlor-5** shows nasal tumors (1 adenocarcinoma, 1 fibrosarcoma at the high dose), however the incidence was not statistically significant and the test was considered to have been performed at too low a dose. For **Chlor-6**, data are not available. As of this time, data do not show nasal turbinate tumors for **Chlor-7.**

2. Stomach tumors

Chlor-3 and **Chlor-2** show tumors in the fundic region of the stomach. Additionally, basal cell tumors have been seen for **Chlor-1**, but statistical significance was not reached. Although 1 gastric carcinoma was observed for **Chlor-6** at the highest dose, the tumor was present in the pyloric region of the rat stomach.

Although **Chlor-3** and **Chlor-2** and possibly **Chlor-1** (at high doses) could be clustered based on the induction of stomach tumors, some limitations exist. Primarily, the diagnosis of the specific tumor types resulting from administration of the various chloroacetanilide herbicides has not been consistent. This is based on the lack of detailed histopathologic analysis of stomach tissue from the various studies conducted. In the case of Chlor-1, "basal cell tumors" were described, while in the case of Chlor-2, "mixed cell tumors" were described. Although initiation/promotion studies with both Chlor-3 and Chlor-2 indicate that both chemicals act as promoters of tumorigenesis through a hormonally mediated, non-genotoxic mechanism, the evidence for support of a common mechanism is not definitive. The carcinomas resulting from Chlor-2 were carcinoids which are unrelated to the proposed gastrin-induced effect. Chlor-3 produced adenocarcinomas and gastric sarcomas which are related to the proposed gastrin-induced effect. Resolution of the specific tumor types involved will aid in determining whether a common mechanism for induction of stomach tumors is operative.

3. Thyroid follicular cell tumors

Thyroid follicular cell tumors have been reported for **Chlor-1, Chlor-2, and Chlor-3** in rats. Additionally, in S-D rats tested with **Chlor-6** at doses of up to 500 ppm in the diet for 104 weeks, the incidence of thyroid neoplasia appeared to be increased, but remained within historical control levels. Thyroid tumors have not been reported for other compounds in the Case Study.

4. Liver histopathology

Examination of liver histopathology indicates that **Chlor-5, Chlor-6 and Chlor-7** are associated with liver tumors in rodents in chronic studies. Chlor-7 has produced a statistically significant trend for hepatocellular adenomas/carcinomas in rats. Chlor-5 produced statistically significant pair-wise increases in liver adenomas and combined adenomas/carcinomas in high-dose female rats and a statistically significant trend for liver tumors in male rats. Chlor-6 produced a statistically significant increase in hepatocellular tumors [adenomas, carcinomas, hepatoblastomas] in high dose male CD-1 mice.

[This case study does not represent a regulatory decision by the EPA. Rather, it attempts to demonstrate a proposed process by which pesticide chemicals can be evaluated.]

B. SAR EVIDENCE

Examination of **SAR relationships** and consideration of common reactive intermediates suggest that only Chlor-1, Chlor-2 and Chlor-3 share a common mechanism of action in several aspects. However, the experimental evidence also suggest that Chlor-5 and Chlor-6 may also share this common mechanism. In addition, depending on the type of tissue involved, all seven pesticides may have a common mechanism of action IF tissue depletion of GSH is a critical factor in toxicity.

Much less is known about the ability of the pesticides in this Case Study to mimic a common biologically active molecule by interfering with the normal homeostasis of the cell to exert toxic action. There is some evidence that several pesticides in this group are capable of inducing mixed-function oxidases; however, there is no evidence to relate any structural moieties or features to their ability to bring about enzyme induction. There is some suggestive evidence that Chlor-2 and Chlor-3 may act as promoters in stomach carcinogenesis; however, there is no evidence that they have any resemblance to the H_2 histamine antagonist and the proton pump inhibitor types of pharmaceutical stomach carcinogens.

C. EVIDENCE FROM PHARMACOKINETICS AND METABOLISM

1. Quinone Imine Formation

Mechanistic studies of nasal tumorigenesis performed with **Chlor-2** and its metabolites indicate that a sulfur metabolite of Chlor-2 is distributed to the nasal turbinates of the rat followed by its subsequent metabolic conversion *in situ* to a reactive quinone imine, 3,5-dialkylbenzoquinone imine (DABQI). DABQI, regarded as the putative toxic metabolite, is considered to bind to cellular protein, resulting in cell death. The ensuing regenerative proliferation of the nasal epithelium is regarded as responsible for fixation of spontaneous mutations and the eventual formation of nasal tumors. Whole body autoradiographic studies indicate that radioactivity from **Chlor-1** is also distributed to the nasal tissues of the rat. Additionally, **Chlor-1** and **Chlor-3** are likely to form the quinone imine, based on the identification of its precursor among the metabolites of the rat. **Chlor-5** appears to have the potential to form a quinone imine, based on the identification of small amounts of the precursor 2,6-dialkyl aniline. Although **Chlor-6** forms the precursor to a quinone imine, there is no evidence that it is distributed to the nasal turbinates in the rat.

2. Microsomal Enzyme Induction

Mechanistic studies of thyroid follicular cell tumorigenesis performed with **Chlor-2** indicate a hormonally-mediated mechanism for thyroid neoplasia. Administration of Chlor-2 results in induction of microsomal hepatic UDPGT activity, which produces increased clearance of thyroid hormone, T_4. Decreased levels of T_4 would result in increased levels of thyroid stimulating hormone (TSH). Increased levels of TSH would result in the hyperplastic and eventually tumorigenic response of the thyroid. Although direct studies have not been conducted with microsomal hepatic UDPGT activity for the other members of this case study, some indirect evidence indicates that they might share Chlor-2's capacity to induce microsomal enzyme activity.

Although mechanistic data for Chlor-1 and Chlor-3 have not been submitted, thyroid tumors are also observed after chronic high dose administration of these chemicals. For Chlor-6, thyroid neoplasia was observed in increased incidence after chronic high dose administration, but was not outside

historical control incidence. For the other current members of this class of chemicals (Chlor-4, Chlor-5, Chlor-7), the production of thyroid tumors has not been observed. Chlor-1, Chlor-3, Chlor-6, and Chlor-7 have produced increases in relative liver weights, coupled in some cases with hepatocellular hypertrophy. These changes are consistent with an induction of microsomal enzymes. Additionally, **Chlor-6** has been found to produce a decreased barbiturate-induced sleeping time in rats (a common indirect test of microsomal enzyme induction).

VI. CONCLUSIONS - GROUPING SCENARIOS

Examination of the above evidence indicates that the Candidate Series of chloroacetanilides can be clustered according to **three** grouping scenarios of varying degrees of validity based on decreasing weight of the evidence.

A. FORMATION OF NASAL TURBINATE TUMORS

Chlor-1, Chlor-2 and Chlor-3 may be grouped together based on a common end-point, a known mechanism of toxicity for this endpoint, and the plausible existence and delivery of the toxic species. This grouping appears to have the strongest support among the three groupings discussed in this section.

Although **Chlor-5** does distribute to the nasal turbinates, and might produce a quinone imine, it is not clear, based on the available data, that an an obvious toxic effect is exerted on the nasal tissue. Although **Chlor-6** does produce a precursor of a quinone imine, there is no data to support its tumorigenicity to the nasal turbinates. Data for **Chlor-4** do not support its inclusion in the group.

B. FORMATION OF THYROID FOLLICULAR TUMORS

Chlor-1, Chlor-2 and Chlor-3 may be grouped together based on a common end-point and a known mechanism of toxicity (microsomal UDPGT induction). However, only data for Chlor-2 exist to confirm that the postulated mechanism of action is indeed responsible for the effect.

In the case of Chlor-1 and Chlor-3, although both produce thyroid tumors in rats, the evidence of UDPGT induction is indirect, at best, and limited to the observation of increases in liver weights and hepatocellular hypertrophy (changes consistent with induction of microsomal enzymes).

Chlor-6 has produced an increased (but not statistically significant) incidence of thyroid neoplasia in mice. Additionally, this incidence was not outside historical control incidence. **Chlor-6** has been found to produce a decreased barbiturate-induced sleeping time in rats, increased liver weights and hepatocellular hypertrophy (all consistent with microsomal enzyme induction). Thus, although **Chlor-6** could qualify as a marginal member of this class, based on the common toxic endpoint although there is no clear evidence for induction of UDPGT.

This second scenario has less certainty than scenario A, above, due to the absence of direct evidence for a causative mechanism of toxicity (e.g., experimental data demonstrating UDPGT induction).

C. FORMATION OF LIVER TUMORS

A third grouping could be attempted to include **Chlor-1, Chlor-7, Chlor-5, and Chlor-6**, based on the production of liver neoplasia in rodents. However, there is no knowledge of a common mechanism of toxicity or of a common toxic species responsible for the effect. This is the least certain of the

scenarios, because a mechanism can not be postulated, although some elements of evidence suggest commonality. EPA does not believe that sufficient evidence is available to support this third grouping scenario.